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International Study on *Artemia**. LII. Incubation of *Artemia* cyst samples at high temperature reveals mixed nature with *Artemia franciscana* cysts

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Abstract

We present evidence that incubation of brine shrimp *Artemia* encysted embryos (cysts) at high temperatures (36–37 °C) suppresses hatching of cysts from a parthenogenetic population, while at the same time allowing cysts of the New World bisexual species (*A. franciscana*) to hatch. This approach is used to verify the presence of contaminating foreign material from the New World in natural cyst populations of Old World *A. parthenogenetica*, *A. sinica*, *A. urmiana* and *A. tunisiana*. We demonstrate that high temperature incubation can be routinely applied to cysts from natural *Artemia* populations occurring adjacent to fish and shrimp hatcheries, to determine the extent and source of contamination.

Keywords: Artemia; Chromocenter; Chromosome; Isozyme; Selection; Temperature

1. Introduction

Large quantities of cysts of the species *Artemia franciscana* (especially from Great Salt Lake, Utah, USA, but also from San Francisco Bay, California, USA, Macau-Brazil and Vin Chau, Vietnam, Van Stappen & Sorgeloos, 1993) are being utilised

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throughout the world in marine fish and shrimp hatcheries. The total annual consumption of *Artemia* cysts is estimated to be about 2000 metric tons, with most hatcheries situated in coastal areas, often in the vicinity of solar saltworks. There is therefore a potential danger that local natural populations and consequently cyst collections of *Artemia* may become contaminated with *A. franciscana*, disseminated from hatchery effluents and/or other methods (e.g. avian dispersal). For this study we selected cyst material collected from the saltworks of Huanghua (Hebei province, People's Republic of China, P.R. China) by the Salt Research Institute (Tanggu, Tianjin province, P.R. China). The Huanghua saltworks are located in the Bohai Bay forming part of the largest area for solar salt production in the P.R. China (Triantaphyllidis et al., 1994). In the early 80s overharvesting of *Artemia* cysts and biomass from the solar saltfields in the Bohai Bay region drastically affected the cyst yields (Xin et al., 1994) as a result of which over 100 tons annually of cysts of *A. franciscana* (Great Salt Lake, Utah, USA) origin were imported to support the local shrimp industy (Tackaert & Sorgeloos, 1991; Salt Research Institute, pers. comm.).

Natural coexistence of zygogenetic and parthenogenetic populations in the same body of water has been determined in a few cases, such as for a salt lake near Cadiz, in Spain (Amat, 1983) and for Lake Urmia, in Iran (Ahmadi et al., 1990). Pronounced differences exist between New World sexual (*A. franciscana*) and Old World sexual (*A. tunisiana*) species with regard to their competitive abilities with parthenogenetic populations (Browne, 1980; Browne & Halanych, 1989), although the outcome of these laboratory experiments has not yet been confirmed in the field. These laboratory tests strongly suggest that *A. franciscana* is likely to outcompete Old World species in many situations. Thus, in the interests of conservation of biodiversity, a fast, sensitive and easily applicable technique which could be used to test whether or not a natural sample of cysts contains *A. franciscana* material would be of value.

Previous work has shown that temperature has a major impact on the hatching ability of cysts and that this response is species specific (Vanhaecke & Sorgeloos, 1989). Moreover, the same study revealed that *A. franciscana* cysts appear to be more resistant to high temperature whereas *A. tunisiana*, *A. persimilis* and *A. parthenogenetica* are more sensitive to it. Hence, it may be possible to take advantage of the partitioning characteristics of high temperatures to check for the presence of *A. franciscana* material in any given sample of cysts.

2. Materials and methods

Artemia cysts from the saltworks of Huanghua (Hebei province, P.R. China) were obtained through the Salt Research Institute (Tanggu, Tianjin province, P.R. China). As controls for electrophoresis and hatching experiments, cysts of *A. franciscana* from San Francisco Bay (California, USA; *Artemia* Reference Center, ARC, No. 1090 and San Francisco Bay Brand Inc., batch No. 2891, ARC No. 1209), *A. sinica* (Inner Mongolia province, P.R. China, ARC No. 1188), *A. tunisiana* (Mégrine, Tunisia, ARC No. 1268), *A. urmiana* (Urmia lake, Iran, ARC No. 1229 and 1230) and *A. parthenogenetica* from Namibia (Swakopmund, Namibia, ARC No. 1186) were used.

For hatching studies, 150 mg cysts were incubated in aerated conical glass tubes containing 100 ml of filtered artificial seawater (Instant Ocean). Na_2CO_3 was added to increase the pH to 8.75 and illumination was at least 2000 lux. The glass tubes were covered to avoid evaporation and immersed in waterbaths of ± 0.1 °C accuracy. The hatching percentage was estimated according to Sorgeloos et al. (1986).

After 48 h cyst incubation, nauplii were separated from the unhatched cysts and transferred to conical tubes for routine culture at 25 ± 1 °C. Animals were cultured at an initial density of one animal per 2 ml of 50 ppt artificial seawater, which was reduced after 8 days to 1 animal per 4 ml. The animals were kept under mild aeration, diffused light and were fed on a mixed diet of the alga *Dunaliella tertiolecta* Butcher and the yeast-based formulated feed LANSY PZ from INVE Aquaculture SA, Belgium (Coutteau et al., 1992). Survival was monitored on days 8, 11 and every day thereafter until all shrimps could be sexed.

Adult animals were isolated from cultures and prepared for allozyme analysis following the procedures described by Abreu-Grobois & Beardmore (1980). Standard horizontal starch gel (12.5%) electrophoresis was applied to whole adult *Artemia* homogenates. Four allozyme loci were used to discriminate between populations: *l*-lactate dehydrogenase (LDH, E.C. No. 1.1.1.27), glucose phosphate isomerase (PGI, E.C 5.3.1.9), phosphogluconate dehydrogenase (6-PGDH, E.C. 1.1.1.44), and NADP-malate dehydrogenase (ME, E.C. 1.1.1.40). Discrimination between the mobilities of electromorphs can readily be used to estimate the probability of correct diagnosis of any one individual (see Ayala & Powell, 1972). In this case for each of the loci used in this study, complete discrimination of 100% is achieved as a result of non-overlapping mobilities between electromorphs of *A. franciscana* and *A. parthenogenetica* (Pilla, 1992) (see Fig. 2). Four loci were used simultaneously to ensure that any lack of enzyme activity in a particular individual would not prevent correct diagnosis.

Chromosome number and chromocenters were studied following the method of Abatzopoulos et al. (1986). Cysts were incubated for hatching at three different temperatures, i.e. 28 ± 1 °C, 35 ± 1 °C and 37 ± 1 °C, for no more than 30 h so as only instar-I nauplii were assayed. At each temperature, at least 75 nauplii were squashed and the determination of the number of chromocenters and/or chromosomes was carried out on ≈ 100 different nuclei per nauplius.

Data (in Tables 1 and 3) were analyzed using *G*-tests for goodness of fit (Sokal & Rohlf, 1981) adjusted by Williams correction factor for small sample size.

3. Results

The effect of the incubation temperature on the hatching percentage of Huanghua cysts is shown in Fig. 1. Starch gel electrophoresis of allozymes reveals that as the incubation temperature is increased, the percentage of the animals that are *A. franciscana* increases (see Table 1). The hatching percentage is severely reduced at 35 °C (5.76 ± 2.15%). *G*-tests revealed that significant differences exist in the frequencies of the two populations and these frequencies are temperature dependent ($p < 0.001$). Nauplii that hatched above 36 °C resulted only in individuals that were *A. franciscana*.

Table 1
Classification of animals from Huanghua population (Hebei province, P.R. of China) hatched out at different temperatures, according to the diagnostic loci 6-PGDH, ME, LDH and PGI as determined by starch gel electrophoresis

Temperature of hatching medium (in °C)	*A. franciscana* type	*A. parthenogenetica* type	Total number of animals	Percent of *A. franciscana* type
28	6	55	61	9.84
33	27	42	69	39.13
34	10	59	69	14.49
35	76	4	80	95.00
36	100	0	100	100.00
37	9	0	9	100.00

The hatching incubation period was 48 h. Animals were reared at 25 ± 1 °C.

Table 2 summarizes the effect of temperature on survival percentage and sex ratio. Survival percentage reduces as temperature increases while the proportion of males increases with temperature. The very low percentage of males at 37 °C can be attributed to the fact that only nine animals reached maturity (14.99% survival); all of them were *A. franciscana*.

It would appear that males favour conspecific females when mating. Thus, for the combined 33° and 34 °C temperatures, 37 out of 138 individuals were of *A. franciscana* type. Of these, 18 would be females (assuming a 1:1 sex ratio) versus 101 parthenogenetic females. Therefore only 15% (19/119) of females were *A. franciscana* and by

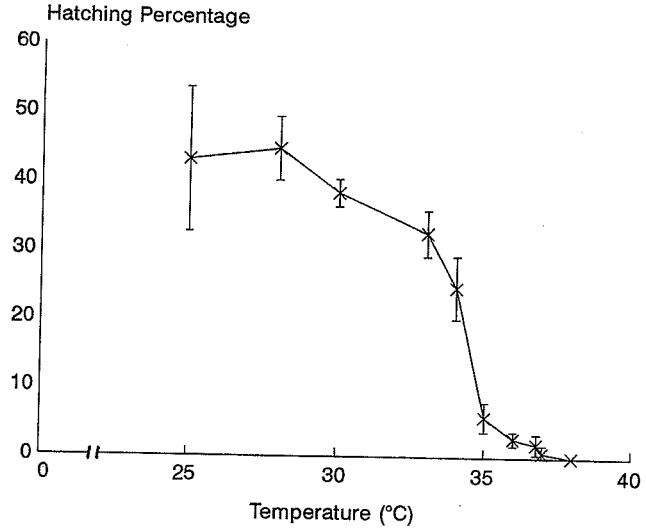

Fig. 1. Effect of temperature on the hatching percentage of cysts from Huanghua saltworks (Hebei province, P.R. China).

Table 2
Survival and sex ratio of animals from Huanghua population (Hebei province, P.R. China) hatched out at different temperatures and cultured at 25 °C

Temperature (°C)	Survival (%)	Sex ratio (% of males)
28	71.17 ± 5.35	< 1
33	66.44 ± 2.68	3.23 ± 2.19
34	68.67 ± 5.03	4.33 ± 1.07
35	32.67 ± 9.45	39.41 ± 10.68
36	34.48 ± 1.38	49.42 ± 17.13
37	14.99	11.11

chance 15% of matings should be conspecific, whereas the value found (four matings out of 11, i.e. 36%) is more than twice that expected by chance alone.

Comparable hatching experiments with cysts from Huanghua, *A. sinica*, *A. tunisiana*, *A. urmiana*, *A. franciscana* and *A. parthenogenetica* from Namibia reveal that *A. franciscana* is the only species in which cysts hatch above 37 °C (*G*-tests, $p < 0.01$, see Table 3).

The population of Huanghua consists mainly of diploid animals (2n = 42). The mean number of chromocenters per nucleus (in *A. franciscana* type) was found to be 13.49 ± 1.31. Table 4 summarizes the results on the number of chromocenters and chromosomes observed in instar-I nauplii hatched at different temperatures.

Thus, both electrophoretic and cytological evidence suggest that the parthenogenetic population from Huanghua is contaminated with the bisexual species *A. franciscana*.

4. Discussion

It appears from the data obtained in this study that temperature has a significant impact on the hatching of *Artemia* cysts. Temperatures above 37 °C for up to 48 h (any longer could result in loss of viability of the nauplii) seem to suppress the hatchability of the parthenogenetic population, allowing preferential hatching of the bisexual species *A. franciscana*.

Vanhaecke & Sorgeloos (1989) demonstrated that the bisexual species *A. persimilis* and *A. tunisiana* resemble parthenogenetic *Artemia* in showing a limited tolerance to high temperatures. Additionally, preliminary experiments carried out with *A. sinica* from Yimeng (Inner Mongolia province, P.R. China) and *A. urmiana* (Urmia lake, Iran) suggest that populations of these species are less tolerant at high temperatures compared with *A. franciscana* (see Table 3). However, differences in temperature tolerance do not necessarily preclude the coexistence of bisexual and parthenogenetic populations.

Although the data on temperature tolerance strongly indicate that differences between one population of *A. franciscana* and another of *A. parthenogenetica* species occur, it would be premature to generalize the results to performance of both species as a whole. Thus, for each species, wide ranges of temperature tolerance exist and may

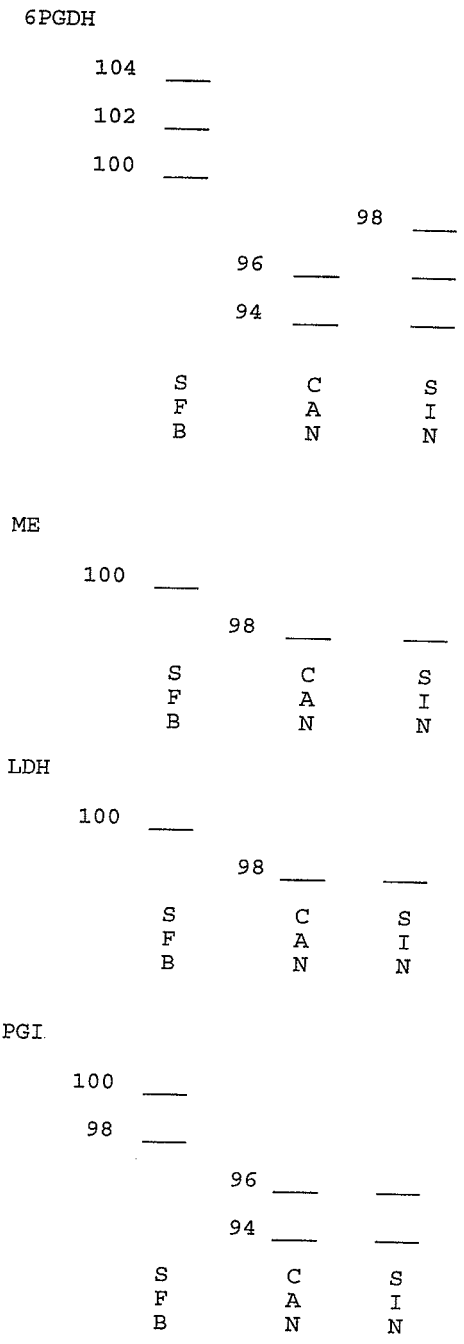

Fig. 2. Allozyme mobilities of the diagnostic loci 6PGDH, ME, LDH and PGI, showing discrimination between *A. franciscana* (SFB) and *A. parthenogenetica* (CAN) from the Huanghua saltworks (Hebei province, P.R. China). A population of the Chinese bisexual species *A. sinica* (from Yuncheng, Shan-xi province) is included for comparison.

Table 3
Effect of temperature on the hatching percentage of different *Artemia* species after 48 hr incubation

Incubation temperature (°C)	Huanghua population	*A. franciscana* (ARC No. 1276)	*A. sinica* (ARC No. 1188)	*A. urmiana* (ARC No. 1229)	*A. persimilis** (ARC No. 410)	*A. tunisiana* (ARC No. 1268)	*A. parthenogenetica* from Namibia (ARC No. 1186)
28 ± 0.1	44.74 ± 4.64	88.24 ± 3.60	81.34 ± 3.85	11.93 ± 3.25	62.70 (25 °C)	66.20 ± 7.84	91.81 ± 1.77
35 ± 0.1	8.31 ± 3.00	77.87 ± 6.50	18.29 ± 3.12	3.64 ± 1.92	–	0.14 ± 0.31	38.85 ± 4.84
36 ± 0.1	2.98 ± 1.08	72.16 ± 3.40	4.39 ± 1.11	1.06 ± 1.25	–	0	4.34 ± 1.23
37 ± 0.1	2.12 ± 2.57	58.34 ± 5.86	0	0	0	0	0
38 ± 0.1	0.23 ± 0.57	7.05 ± 1.97	0	0	–	0	0

* Results from Vanhaecke & Sorgeloos, 1989.

Table 4
Percentage of nauplii with or without chromocenters and chromosome numbers in instar-I nauplii hatched out at different temperatures

Temperature (°C)	Instar-I nauplii with/without chromocenters (%)			Chromosome number (Nauplii %)		Number of instar-I nauplii assayed
	+[a]	−[b]	?[c]	Diploid	Polyploid	
28 ± 1	0	97.3	2.4	94.6	5.4	75
35 ± 1	89.2	8.4	2.4	100.0	0	83
37 ± 1	97.9	0	2.1	100.0	0	97

[a] Distinct chromocenters of *A. franciscana* type.
[b] Absence of chromocenters.
[c] Non identifiable.

well overlap. This is especially true for both *A. parthenogenetica*, for which large intraspecific differences in life-history traits have been reported (Browne & Hoopes, 1990) and for *A. franciscana*, a supraspecies composed of a variety of populations in different stages of incipient speciation (Browne and Bowen, 1991).

Heterogamic mating (or "sperm robbing") has been observed in mixed *Artemia* populations (Browne, 1980; Browne & Halanych, 1989). In addition, *A. franciscana* and *A. tunisiana* males have been seen to clasp females of the anostracans *Branchinella* (Mura, 1987) and *Branchinecta* (Belk & Serpa, 1992). Nevertheless, Browne et al. (1991) and Pilla (1992) reported results showing preferential pairing between males and females of *Artemia* belonging to the same population and species. Although observation of pairing couples in the Huanghua population suggests that males favoured conspecific females, it is quite possible that males which were clasping conspecific females had already mated with asexual females, since no periodical observations were made. Further, a skewed sex ratio (as observed in the higher temperatures) would also bias the proportion of conspecific matings. However, it would seem that in a mixed population consisting of an asexual and a sexual species the asexual species may have a competitive advantage (albeit small) over its sexual counterpart, by preventing some of the males from mating with females of their own species (since asexual females do not require males in order to reproduce).

Since chromocenters (heterochromatic blocks in the resting nuclei, Barigozzi & Baratelli Zambruni, 1982; Barigozzi et al., 1984) are present in the nuclei of New World species *A. franciscana* and *A. persimilis* but absent from the parthenogenetic populations and the Old World sexual species *A. tunisiana*, *A. urmiana* and *A. sinica* (Abreu-Grobois, 1983; Abatzopoulos et al., 1986; Pilla, 1992) they can serve as an additional tool for the characterization of a cyst sample.

Vanhaecke & Sorgeloos (1989) suggested that "the criterion of temperature tolerance of cyst hatching appears to be a helpful tool to carry out a quick screening and preliminary classification of *Artemia* strains". Our results confirm that temperature tolerance could be used to determine whether or not there is contamination with cysts of *A. franciscana* origin but not with other bisexual species. This is because temperature

tolerance does not distinguish between parthenogenetic populations and bisexual populations other than *A. franciscana*. Even so, this method could be of use as *A. franciscana* is being used all over the world and so far it is the main commercially available source of cysts for the fast expanding aquaculture industry.

Additionally, this high temperature screening could be used to purify cyst samples thought to be contaminated with *A. franciscana*, should a purer sample of a natural population be required. Although it is probably very difficult to estimate the exact degree of contamination, this method could, with refinement, lead to a more quantitative estimate of the extent of contamination.

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